Sabouraudia (1976), **14**, 275–280

ISOLATION OF A SPECIFIC ANTIGEN WITH ALKALINE PHOSPHATASE ACTIVITY FROM SOLUBLE EXTRACTS OF *PARACOCCIDIOIDES BRASILIENSIS*

L. A. Yarzabal*, Suzanne Andrieu†, D. Bout* and Frida Naquira*

**Service d'Immunologie et de Biologie Parasitaire* (*Directeur*: *Professeur* A. Capron), *Faculté de Médecine et Institut Pasteur, Lille*, 59000, *France*

† *Unité INSERM U42 de Biologie et d'Immunologie Parasitaires et Fongiques* (*Directeur*: *Professeur* J. Biguet), *Villeneuve-d'Ascq*, 59650, *France*

A specific antigen of *Paracoccidioides brasiliensis* was isolated from a metabolic extract of the fungus.

The extraction was made by specific adsorption to and subsequent elution from a column containing a cross linked polymer to which the antibodies of a monospecific rabbit serum had been covalently attached.

The purity of the final product was demonstrated by immunodiffusion analysis of the eluate using immune serum produced in a sensitized rabbit.

The purified antigen was shown to have a cationic electrophoretic mobility and alkaline phosphatase activity.

Earlier studies have demonstrated that the majority of the sera extracted from patients with paracoccidiomycosis formed specific antigen-antibody precipitating systems when reacting with crude soluble extracts of *Paracoccidioides brasiliensis* in immunoelectrophoresis (Yarzabal, 1971). This system appears as opaque bands with a surface distributed 2/3 or more in the cathodic area of immunoelectrophoretic diagrams. One of them, designated as band E, possesses a particular importance because of the frequence with which it is formed by the sera of confirmed infected patients and the specificity which it gives to the immunologic diagnosis of paracoccidioidomycosis.

In previous research it was postulated that the antigen responsible for the formation of this precipitating system was a protein with cationic mobility having alkaline phosphatase activity (Yarzabal, Biguet, Vaucelle, Andrieu, Torres & Da Luz, 1973).

The purpose of the present research was to develop a procedure for the separation of the specific antigens of the fungus and to isolate the antigen with the alkaline phosphatase activity.

Materials and Methods

Organisms and Conditions of Culture

The strains used were: *Aspergillus fumigatus* (supplied by J. L. Longbottom); *Blastomyces dermatitidis* G. R. A. (provided by E. S. McDonough); *Histoplasma capsulatum* IHM‡ 1524; *H. duboisii* Sekou (isolated in the "Laboratoire de Microbiologie" of St. Louis Hospital, Paris); *H. farciminosum* 4033 (supplied by P.K.C. Austwick)

‡IHM = Instituto De Higiene De Montevideo (URUGUAY).

and *P. brasiliensis* IHM 1572. All strains are stocked in INSERM Unity U42. The medium used for antigen production was the Sabouraud's dextrose broth.

For the preparation of cultures, the mycelial growth from a 15-days-old Sabouraud's broth was homogenized in a Virtis homogenizer for 5 min at 4°C, transferred to a flask with 200 ml of Sabouraud's broth and incubated at 25°C for 12 weeks (with the exception of *A. fumigatus* which was incubated at 37°C for 4 weeks only).

Preparation of Crude Antigens

At the end of the period of incubation, the cultures were checked for contamination and cellular elements were extracted by filtration. The cultures were then centrifuged at 2000 g for 30 min in a refrigerated centrifuge to remove cellular debris. The remnant fluids were dialysed against distilled water for 48 h at 4°C with two daily changes of water, after which the dialyzed cultures were lyophilized.

Production of Antisera

Immune sera against crude antigens were prepared as proposed by Yarzabal *et al.* (1973) by mixing 40 mg of each fungus antigen diluted in 5 ml of saline with an equal volume of Freund's complete adjuvant. Hyperimmunization was attained by injecting adult rabbit (weighing approx. 3 kg each), with 1 ml of homogenate, in the axillary region, at weekly intervals for 10 weeks.

Antisera to the specific antigen with alkaline phosphatase activity were obtained by the procedure described by Goudie, Horne & Wilkinson (1966) as modified by Bout, Fruit & Capron (1974). Briefly, the precipitation line consisting of the chosen antigen combined with rabbit antibody was identified in an immunoelectrophoretic slide performed with an anti-crude *P. brasiliensis* serum, absorbed with heterologous fungus extracts sharing common antigens.

To absorb the rabbit immune serum anti-*P. brasiliensis* (RIS anti-Pb) 10 mg of each heterologous fungus dry extract was added to 1 ml of antiserum. The precipitation was allowed to form at 37°C for 2 h, then at 4°C for 18 h. The mixture was centrifuged to pack the precipitate and the supernatant fluid was separated. This procedure was repeated until precipitate was no longer detectable. The absorbed serum (Abs RIS anti-Pb) was tested by immunodiffusion.

After 48 h washing with Phosphate buffered saline, the zone of agar containing the appropriate band was excised. The agar which contained the precipitate was then broken up to 0·5 ml of saline and emulsified in 0·5 ml of Freund's complete adjuvant. The antigen-antibody-adjuvant mixture was injected into the dermis of one rabbit according to the method of Vaitukaitis, Robbins, Nieschlag & Ross (1971). Blood samples were obtained 15 days later.

Chemical Coupling of Antibodies

Antibodies formed against *P. brasiliensis* specific antigen were fixed to a solid support by the procedure of Axen, Porath & Ernback (1967). Fifteen mg of rabbit monospecific IgG were dissolved in 0·1 M $NaHCO_3$—0·5 M NaCl buffer and added to 3·5 ml of CNBr-activated Sepharose*. This mixture was stirred gently at 25°C for 2 h after which it was washed extensively with bicarbonate buffer and suspended in a 15 ml chromatographic column.

* Pharmacia, Uppsala, Sweden.

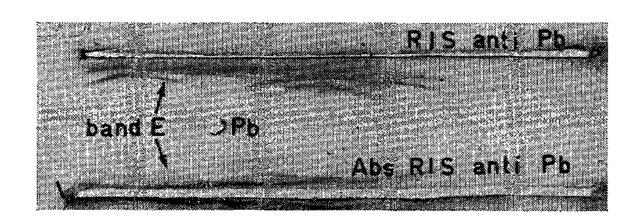


Figure 1.—Immunoelectrophoresis of crude *P. brasiliensis* (Pb) antigen using a rabbit immune serum anti-*P. brasiliensis* (RIS anti-Pb), and the same immune serum absorbed with heterologous fungus antigens (Abs RIS anti-Pb).

Extraction of the Antigen

The crude *P. brasiliensis* extract was added to the washed Sepharose and the column was connected to a LKB fraction collector.

After washing with 0·5 M NaCl the fixed antigen was eluted by acid dissociation with a 0·2 M glycine—HCl—0·5 M NaCl, pH 2·8 buffer.

Immunochemical Analysis

Four precipitation reactions were employed for the analysis of the antigens and the antisera: (i) the Abelev (1960) and Ouchterlony (1948) variants of the double diffusion in gel; (ii) the immunoelectrophoretic analysis of Grabar & Williams (1953) (iii) the bidimensional immunoelectrophoresis described by Axelsen (1971); and (iv) the electrosyneresis of Bussard (1959) as modified by Conti-Diaz, Somma-Moreira, Gezuele, Gimenez, Pena & Mackinnon (1973).

The enzymatic activity was revealed according to the procedure described by Uriel (1963). Alkaline phosphatase activity was investigated by incubation of the dried immunodiffusion or immunoelectrophoretic slides for two hours at 25°C in a substrate of ASMx phosphoric acid* (5·0 mg) diluted in dimethylformamide (0·5 ml), and mixed with $MgSO_4$ $7H_2O$ 0·25 M (1·0 ml) and Tris buffer 0·05 M pH 8·4 (19 ml). Fast Garnet GBC* (20 mg) diluted in sodium acetate buffer 0·2 M pH 4·8 (20 ml) was used as a coupler. The bands possessing the alkaline phosphatase activity developed a rose color. To control the purity of the isolated antigen, 0·5 ml of immunoadsorbent eluate was injected intradermically to a rabbit (Vaitukaitis *et al.*, 1971) and the immune serum was tested in immunoprecipitation tests.

Results

As shown in figures 1 and 2, the absorption of rabbit antiserum prepared with crude extract of *P. brasiliensis* strain IHM 1572, with culture extracts of *A. fumigatus*, *B. dermatitidis*, *H. capsulatum*, *H. duboisii* and *H. farciminosum* eliminated the antibodies directed against the common antigens.

By means of intradermic injection to a rabbit of E band taken from a bidimensional immunoelectrophoretic slide done with absorbed antiserum and *P. brasiliensis* crude antigen, antiserum (RIS anti-band E) was produced which reacts with two (2) of the species specific cationic antigens (fig. 3).

One of them is the protein supporting alkaline phosphatase enzymatic activity,

* Sigma Chemical Company, St Louis, Missouri.

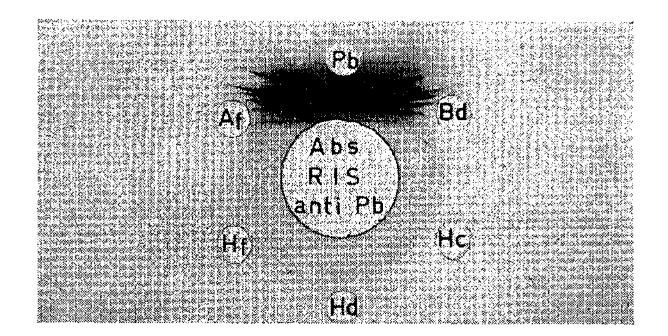


Figure 2.—Immunodiffusion of the absorbed immune serum against crude extracts of *P. brasiliensis* (Pb), *B. dermatitidis* (Bd), *H. capsulatum* (Hc), *H. duboisii* (Hd), *H. farciminosum* (Hf) and *A. fumigatus* (Af).

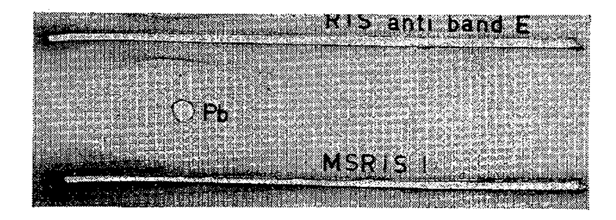


Figure 3.—Immunoelectrophoresis of the rabbit immune serum and anti-band E (Ris anti-band E) and of the "monospecific" rabbit immune serum anti-E_1 antigen (MSRIS I), against the crude antigen of *P. brasiliensis* (Pb).

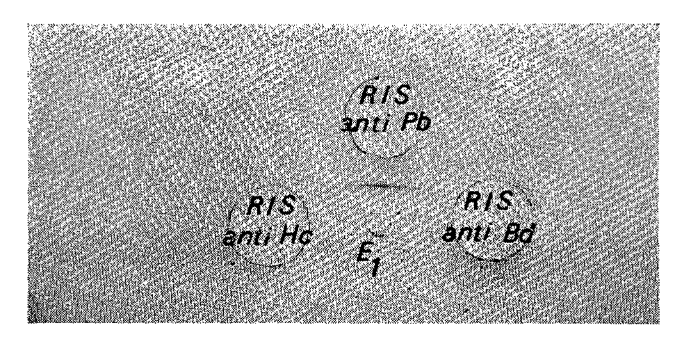


Figure 4.—Immunodiffusion of purified E_1 antigen against rabbit immune sera anti-crude *P. brasiliensis* (Pb), *B. dermatitidis* (Bd), and *H. capsulatum* (Hc) extracts.

and will be designated E_1. The other, which lacks this enzyme, is located in the same zone in the diagram and will be designated E_2. Using "bispecific" antiserum against *P. brasiliensis* crude antigen, a precipitant system corresponding to antigen E_1 was extracted. This system, injected intradermically into another rabbit, elicited a "monospecific" antiserum (MS RIS I) against antigen E_1 (fig. 3).

Chromatography of *P. brasiliensis* crude antigen through the immunoadsorbent which contained the immunoglobulin anti-antigen E_1 liberated, after elution, a highly purified product.

The antigen extracted by this means formed only one precipitation band with anti-*P. brasiliensis* crude antigen antiserum, and did not react with the antisera prepared against the crude antigens of *B. dermatitidis* and *H. capsulatum* (fig. 4).

The immune serum obtained by injecting the isolated antigen confirmed its purity by revealing only one component in crude extracts of *P. brasiliensis*. The alkaline phosphatase activity was easily revealed in the purified antigen by following the method described by Uriel (1963). After the incubation with the substrate the band formed with immune serum, developed a rose color in the presence of Fast Garnet.

Discussion

The results of this study showed that the immunoadsorption method can be successfully adapted to the isolation of highly purified specific antigens of *P. brasiliensis*.

Previous attempts for separating such substances from metabolic extracts of the fungus by ion exchange chromatography (Torres, Nieto, Da Luz, Lopez Lemes, Guisantes, Josef, Coch & Yarzabal, 1974) did not produce antigens free from "contaminating" fractions common to other pathogenic fungi but the resultant antigen was less complex and more specific than the original extract.

The progress attained in the isolation of pure antigenic substances from complex extracts of parasitic origin by means of the immunoadsorption procedure (Bout, Fruit & Capron, 1973); Bout *et al.*, 1974) induced us to use such method for the isolation of the specific antigen with alkaline phosphatase activity identified in soluble extracts of *P. brasiliensis*.

The preparation of the "monospecific" antiserum was a fundamental step of the isolation procedure. It was obtained by intradermal immunization of rabbits with the appropriate antigen-antibody system excised from electrosyneresis and bidimensional immunoelectrophoretic slides. The final product is an immunologically pure substance which conserves its original immunochemical properties. The experimental procedure presented here may favor the isolation of other important *P. brasiliensis* specific antigens. Such separation could provoke unpredictable conceptual advances in the knowledge of the physiology and the metabolic pathways of *P. brasiliensis*. On the other hand, obtaining antigens free from common antigenic substances may favor future progress in immunodiagnosis and in the study an understanding of the immune response of paracoccidioidomycotic patients. Our laboratories are presently conducting further investigation on serological reactivity of purified antigen.

Resume

Grâce à l'utilisation d'un immunsérum unispécifique, la chromatographie d'affinité nous a permis l'isolement d'un antigène spécifique de *Paracoccidioides brasiliensis* possédant une activité enzymatique de type phosphatase alcaline.

La pureté de l'antigène ainsi isolé a été contrôlée en immunodiffusion.

Acknowledgements

The authors wish to thank Mrs. Réjane Popeye and Mr. Didier Deslee for technical assistance.

This work was supported by the "Institut National de la Santé Publique" (INSERM) and the "Centre National de la Recherche Scientifique" (CNRS-ERA 422).

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